Polymorphisms of Hepatitis C Virus Non-Structural Protein 5A and Core Protein and Clinical Outcome of Pegylated-Interferon/Ribavirin Combination Therapy

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Key Words

Hepatitis C virus • Non-structural protein 5A • Interferon/ribavirin resistance-determining region • Interferon sensitivity-determining region • Core protein • Sustained virological response • Prediction

Abstract

Objective: Hepatitis C virus (HCV genome) polymorphisms are thought to influence the outcome of pegylated-interferon/ribavirin (PEG-IFN/RBV) therapy. This study aimed to examine non-structural protein 5A (NS5A) polymorphisms, e.g. IFN/RBV resistance-determining region (IRRDR) and IFN sensitivity-determining region (ISDR), and core protein polymorphism as predictive therapeutic markers. **Methods:** Pre-treatment sequences of NS5A and core regions were analyzed in 68 HCV-1b-infected patients treated with PEG-IFN/RBV. **Results:** Of 24 patients infected with HCV having an IRRDR with 6 or more mutations (IRRDR $\geq$ 6), 18 (75%) patients achieved sustained virological response (SVR), whereas only 11 (25%) of 44 patients infected with HCV having IRRDR $\leq$ 5 did. IRRDR $\geq$ 6 was significantly associated with SVR ($p < 0.0001$). On the other hand, ISDR $\geq$ 2 was significant-

ly associated with relapse (either before [breakthrough] or after end-of-treatment response [ETR-relapse]) ($p < 0.05$) and a point mutation of the core protein from Arg to Gln at position 70 (Gln⁷⁰) was significantly associated with null-response ($p < 0.05$). Multivariate analysis identified IRRDR $\geq$ 6 as the only viral genetic factor that independently predicted SVR. **Conclusion:** NS5A (IRRDR and ISDR) and core protein polymorphisms are associated with the outcome of PEG-IFN/RBV therapy for chronic hepatitis C. In particular, IRRDR $\geq$ 6 is a useful marker for prediction of SVR.

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Introduction

Hepatitis C virus (HCV) is the major cause of chronic liver diseases worldwide [1]. As a consequence of the long-term persistence of chronic hepatitis C, the number of patients with hepatocellular carcinoma is expected to increase further over the next 20 years [2]. To reduce the impact of this worldwide health problem, efficient treatment is required. Currently, a combination therapy of pegylated-interferon- α and ribavirin (PEG-IFN/RBV) is a

standard treatment for chronic hepatitis C [3]. However, this therapy is sometimes difficult to tolerate and results in a sustained virological response (SVR) in only ~50% of patients, especially those infected with the most resistant genotypes, HCV-1a and HCV-1b [3]. Given the considerable side effects, the possibility of discontinuation and the high cost of this treatment, prediction of treatment outcome is needed. An expanded range of predictors may assist clinicians and patients in more accurately assessing the likelihood of an SVR and thus in making more informed treatment decisions [4].

Since the HCV genotype is one of the major factors affecting the IFN-based therapy response, IFN resistance is, at least partly, genetically encoded by HCV itself [5]. In this context, non-structural protein 5A (NS5A) has been widely discussed for its correlation with IFN responsiveness. Enomoto et al. [6] proposed that sequence variations within a region in NS5A spanning from amino acids (aa) 2,209 to 2,248, called the IFN sensitivity-determining region (ISDR), is correlated with IFN responsiveness. Recently, we identified a new region near the C-terminus of NS5A spanning from aa 2,334 to 2,379, which we referred to as the IFN/RBV resistance-determining region (IRRDR) [7]. The degree of sequence variation within IRRDR was significantly associated with the clinical outcome of PEG-IFN/RBV combination therapy. On the other hand, prediction of SVR by aa substitutions within the core protein in Japanese patients infected with HCV-1b has also been proposed [8, 9]. In multivariate analysis, the criterion of double-wild core, presence of Arg at position 70 and Leu at position 91 (Arg⁷⁰/Leu⁹¹), was identified as an independent SVR predictor.

This study aimed to examine NS5A polymorphisms, including those in IRRDR and ISDR, and core polymorphism as predictive markers for HCV treatment outcome. The core protein with Arg⁷⁰/Leu⁹¹ was defined as wild-core while the other patterns as non-wild-core. The possible correlation of either Arg⁷⁰ alone or Leu⁹¹ alone with the clinical outcome of PEG-IFN/RBV therapy was also examined.

Patients and Methods

Patients

A total of 68 patients seen at Kobe Asahi Hospital in Kobe, Japan, who were chronically infected with HCV-1b, with diagnoses based on anti-HCV antibody detection and HCV-RNA detection, were enrolled in the study. HCV subtype was determined as according to the method of Okamoto et al. [10]. Patients were treated with PEG-IFN α -2b (Pegintron®; Schering-Plough, Kenilworth,

N.J., USA) (1.5 μ g/kg b.w., once weekly, s.c.) and RBV (Rebetol®; Schering-Plough) (600–800 mg daily, per os), according to a standard treatment protocol for Japanese patients established by a hepatitis study group of the Ministry of Health, Labor and Welfare, Japan. All patients received >80% of scheduled dosage of PEG-IFN and RBV. Serum samples were collected from the patients at intervals of 4 weeks before, during and after the treatment, and tested for HCV RNA and core antigen titers as reported previously [11].

The study protocol was approved beforehand by the Ethic Committee in Kobe Asahi Hospital, and written informed consent was obtained from each patient prior to the treatment.

Sequence Analysis of HCV NS5A and Core

HCV RNA was extracted from 140 μ l of serum using a commercially available kit (QIAmp viral RNA kit; Qiagen, Tokyo, Japan). Amplification of full-length NS5A and core regions of the HCV genome were performed as described elsewhere [7, 11, 12]. The sequences of the amplified fragments of NS5A and core regions were determined by direct sequencing. The aa sequences were deduced and aligned using Genetyx Win software version 7.0 (Genetyx Corp., Tokyo, Japan).

Statistical Analysis

Statistical differences in the patients' baseline parameters according to the degree of IRRDR polymorphism were determined by Student's *t* test for numerical variables and Fisher's exact probability test for categorical variables. Likewise, statistical differences in treatment responses according to NS5A and core polymorphisms were determined by Fisher's exact probability test. Kaplan-Meier HCV survival curve analysis was performed based on serum HCV-RNA positivity data during the treatment period (48 weeks) according to NS5A and core polymorphisms. The data obtained were evaluated by the log-rank test. Uni- and multivariate logistic analyses were performed to identify variables that independently predicted the treatment outcome. Variables with a *p* value of <0.1 in univariate analysis were included in a multivariate logistic regression analysis. The odds ratios and 95% confidence intervals (95% CI) were also calculated. All statistical analyses were performed using SPSS version 16 software (SPSS Inc., Chicago, Ill., USA). Unless otherwise stated, a *p* value <0.05 was considered as statistically significant.

Nucleotide Sequence Accession Numbers

The sequence data reported in this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession numbers AB285035 through AB285081, AB354116 through AB354118, and AB518774 through AB518861.

Results

Patients' Responses to PEG-IFN/RBV Combination Therapy

Among 68 patients enrolled in this study, HCV-RNA negativity was achieved by 8 (12%) patients at week 4 (rapid virological response [RVR]), 36 (53%) patients at week 12 (early virological response [EVR]), 47 (69%) patients at

Table 1. Proportions of various virological responses of patients treated with PEG-IFN/RBV

Virological response	Proportion, patients	
	n/total	%
RVR	8/68	12
EVR	36/68	53
ETR	47/68	69
SVR	29/68	43
Non-SVR	39/68	57
Null-response	17/68	25
ETR-relapse	18/68	26
Breakthrough	4/68	6

PEG-IFN/RBV = Pegylated-interferon/ribavirin; RVR = rapid virological response; EVR = early virological response; ETR = end-of-treatment response; SVR = sustained virological response.

week 48 (end-of-treatment response [ETR]) and 29 (43%) patients at week 72 (SVR) (table 1). A total of 39 patients (57%) failed to achieve SVR and they were referred to as non-SVR. Non-SVR can be further divided into three categories: (i) null-response, which is defined by continued presence of serum HCV RNA during the entire period of the treatment and follow-up; (ii) breakthrough, defined as transient disappearance of HCV RNA followed by its re-appearance before the end of the 48-week treatment, and (iii) ETR-relapse, defined by reappearance of HCV RNA after ETR has been achieved. Seventeen (25%) patients were null-response while 18 (26%) and 4 (6%) patients were ETR-relapse and breakthrough, respectively (table 1).

Correlation between NS5A Polymorphism and Treatment Responses

Using a receiver operating characteristic curve analysis, 6 mutations in IRRDR were previously estimated as an optimal cutoff number of mutations for SVR prediction [7]. Initially the correlation between the patients' demographical, hematological, biochemical and virological baseline parameters and the degree of IRRDR polymorphism was examined. This analysis revealed that patient's sex was the only factor that significantly correlated to the degree of IRRDR polymorphism since 49% (17/35) of males were infected with HCV isolates having IRRDRs with 6 mutations or more ($IRRDR \geq 6$) compared to 21% (7/33) of females ($p = 0.02$) (table 2). HCV-RNA titers or HCV core antigen titers did not differ significantly between patients infected with HCV isolates of $IRRDR \geq 6$ and those of $IRRDR \leq 5$.

Next, the possible correlation between IRRDR polymorphism and the ultimate treatment responses was examined. Among 24 patients infected with HCV isolates of $IRRDR \geq 6$, 18 (75%), 6 (25%), 3 (12.5%) and 3 (12.5%) patients were SVR, non-SVR, null-response and relapse (ETR-relapse *plus* breakthrough), respectively (table 3). By contrast, among 44 patients infected with HCV isolates of $IRRDR \leq 5$, 11 (25%), 33 (75%), 14 (32%) and 19 (43%) patients were SVR, non-SVR, null-response and relapse (ETR-relapse *plus* breakthrough), respectively. The proportions of different treatment responses among HCV isolates with $IRRDR \geq 6$ and $IRRDR \leq 5$ were significantly different. Furthermore, patients infected with HCV isolates with Ala at position 2360 (Ala²³⁶⁰) in IRRDR had a more significant likelihood of SVR than those infected with HCV isolates with non-Ala²³⁶⁰, who tended to be non-SVR, in particular null-response (table 3; fig. 1).

As the IRRDR polymorphism was closely correlated with the ultimate treatment responses, it was also significantly correlated with the on-treatment responses, in particular EVR and ETR (table 4). However, there was no significant correlation between the IRRDR polymorphism and RVR. Also, the presence of Ala²³⁶⁰ was correlated significantly with ETR.

Regarding the analysis of ISDR polymorphism and its correlation to the treatment responses, first, the criterion of ISDR with 4 mutations or more ($ISDR \geq 4$), the initial criterion of IFN responsiveness proposed by Enomoto et al. [6] was tested. Since the prevalence of $ISDR \geq 4$ was only 9% (6/68) of all isolates analyzed, this criterion did not significantly correlate with the treatment responses (data not shown). Next, the correlation between the treatment responses and ISDR mutations at a cutoff point of 2 mutations, a newly proposed ISDR criterion of PEG-IFN/RBV responsiveness [13, 14] was tested. Although there was no significant difference in the proportions of SVR and non-SVR between HCV isolates with ISDR of 2 mutations or more ($ISDR \geq 2$) and those of $ISDR \leq 1$, a small but significant difference in the proportions of SVR and relapse (ETR-relapse *plus* breakthrough) was observed between $ISDR \geq 2$ and $ISDR \leq 1$ (table 3). Interestingly, ISDR polymorphism was the only virological factor examined in this study that showed a significant correlation with RVR (table 4). However, this correlation disappeared when further time points of treatment course, such as EVR and ETR, were considered.

Table 2. Correlation between IRRDR polymorphism and patients' demographic characteristics

Factor	IRRDR $\geq$ 6	IRRDR $\leq$ 5	p value
Age, mean $\pm$ SD	58.71 $\pm$ 8.44	59.61 $\pm$ 10.30	0.71
Sex, male/female	17/7	18/26	0.02
Body weight, kg	59.87 $\pm$ 9.56	58.20 $\pm$ 11.92	0.56
Platelets, $\times 10^4/\text{mm}^3$	17.22 $\pm$ 5.5	14.96 $\pm$ 4.71	0.16
Hemoglobin, g/dl	14.25 $\pm$ 1.48	13.55 $\pm$ 1.77	0.11
γ -GTP, IU/l	49.50 $\pm$ 44.29	55.60 $\pm$ 65.60	0.69
GPT, IU/l	47.54 $\pm$ 33.09	49.33 $\pm$ 34.78	0.84
HCV-RNA, KIU/ml	2,070.21 $\pm$ 1,720.27	2,038.57 $\pm$ 1,963.05	0.95
HCV core antigen, fmol/l	6,750.87 $\pm$ 6,859.82	9,320.52 $\pm$ 10,636.48	0.30

IRRDR = Interferon/ribavirin resistance-determining region; γ -GTP = γ -guanosine triphosphate; GPT = glutamic pyruvate transaminase.

Table 3. Correlation between NS5A and core protein polymorphisms and ultimate virological responses of patients treated with PEG-IFN/RBV

Protein	Factor	Total ^a	SVR ^b	Non-SVR	Null-response	Relapse (ETR-relapse <i>plus</i> breakthrough)	p value		
							SVR vs. non-SVR	SVR vs. null-response	SVR vs. relapse (ETR-relapse <i>plus</i> breakthrough)
NS5A	IRRDR $\geq$ 6	24	18 (75) ^c	6 (25)	3 (12.5)	3 (12.5)	<0.0001	0.005	0.0006
	IRRDR $\leq$ 5	44	11 (25)	33 (75)	14 (32)	19 (43)			
	Ala ²³⁶⁰	18	12 (67)	6 (33)	1 (5)	5 (28)	0.026	0.016	0.2
	Non-Ala ²³⁶⁰	50	17 (34)	33 (66)	16 (32)	17 (34)			
	ISDR $\geq$ 2	18	10 (56)	8 (44)	6 (33)	2 (11)	0.27	1.0	0.048
	ISDR $\leq$ 1	50	19 (38)	31 (62)	11 (22)	20 (40)			
Core	Wild-core (Arg ⁷⁰ /Leu ⁹¹)	33	18 (55)	15 (45)	5 (15)	10 (30)	0.1	0.07	0.27
	Non-wild-core	35	11 (31)	24 (69)	12 (34)	12 (34)			
	Gln ⁷⁰	21	5 (24)	16 (76)	8 (38)	8 (38)	0.06	0.04	0.19
	Non-Gln ⁷⁰	47	24 (51)	23 (49)	9 (19)	14 (30)			
	Met ⁹¹	19	7 (37)	12 (63)	5 (26)	7 (37)	0.59	0.74	0.75
	Non-Met ⁹¹	49	22 (45)	27 (55)	12 (24)	15 (31)			

SVR = Sustained virological response; ETR = end-of-treatment response; IRRDR = interferon/ribavirin resistance-determining region; Ala²³⁶⁰ = alanine at position 2360; ISDR = interferon sensitivity-determining region; Arg⁷⁰ = arginine at position 70; Leu⁹¹ = leucine at position 91; Gln⁷⁰ = glutamine at position 70; Met⁹¹ = methionine at position 91.

^a Total number of isolates with a given factor.
^b Number of SVR, non-SVR, null-response or relapse (ETR-relapse *plus* breakthrough) cases with a given factor.
^c Values in parentheses are percentages.

Correlation between Core Polymorphism and Treatment Responses

Recently, it was reported that polymorphism at positions 70 and/or 91 of the core protein of HCV-1b correlates with and predicts the treatment outcome of Japanese patients treated with PEG-IFN/RBV combination therapy

[8, 9]. We aimed to test the consistency of this observation among our patient cohort. The result revealed that among 33 patients infected with HCV isolates of wild-core (Arg⁷⁰/Leu⁹¹), 18 (55%), 15 (45%), 5 (15%) and 10 (30%) patients were SVR, non-SVR, null-response and relapse (ETR-relapse *plus* breakthrough), respectively (table 3; fig. 1). On

Fig. 1. Sequence alignment of the core protein (aa 65–96) and IRRDR of NS5A obtained from pretreated sera in patients infected with HCV-1b. The consensus (Cons) sequence is shown at the top. Amino acids at positions 70 and 91 of the core protein, and position 2360 of NS5A are shown in boldface.

the other hand, of 35 patients infected with HCV isolates of non-wild-core, 11 (31%), 24 (69%), 12 (34%) and 12 (34%) patients were SVR, non-SVR, null-response and relapse (ETR-relapse *plus* breakthrough), respectively. Thus, there was no significant correlation between wild-core and SVR or non-SVR ($p = 0.1$). However, a single mutation at posi-

tion 70 (Gln⁷⁰ vs. non-Gln⁷⁰) was significantly correlated with treatment outcome (SVR vs. null-response; $p = 0.04$).

As for the on-treatment responses, wild-core (Arg⁷⁰/Leu⁹¹) was significantly correlated with EVR and ETR, whereas Gln⁷⁰ was correlated with non-EVR and non-ETR (table 4).

Table 4. Correlation between NS5A and core protein polymorphisms and on-treatment virological responses of patients treated with PEG-IFN/RBV

Protein	Factor	Total ^a	RVR ^b	Non-RVR	EVR	Non-EVR	ETR	Non-ETR	p value		
									RVR vs. non-RVR	EVR vs. non-EVR	ETR vs. non-ETR
NS5A	IRRDR $\geq$ 6	24	5 (21) ^c	19 (79)	17 (71)	7 (29)	21 (87)	3 (13)	0.12	0.04	0.026
	IRRDR $\leq$ 5	44	3 (7)	41 (93)	19 (43)	25 (57)	26 (59)	18 (41)			
	Ala ²³⁶⁰	18	4 (22)	14 (78)	13 (72)	5 (28)	16 (89)	2 (11)	0.19	0.1	0.04
	Non-Ala ²³⁶⁰	50	4 (8)	46 (92)	23 (46)	27 (54)	31 (62)	19 (38)			
	ISDR $\geq$ 2	18	6 (33)	12 (67)	9 (50)	9 (50)	11 (61)	7 (39)	0.003	0.79	0.39
	ISDR $\leq$ 1	50	2 (4)	48 (96)	27 (54)	23 (46)	36 (72)	14 (28)			
Core	Wild-core (Arg ⁷⁰ /Leu ⁹¹)	33	5 (15)	28 (85)	23 (70)	10 (30)	28 (85)	5 (15)	0.47	0.009	0.009
	Non-wild-core	35	3 (9)	32 (91)	13 (37)	22 (63)	19 (54)	16 (46)			
	Gln ⁷⁰	21	2 (10)	19 (90)	6 (29)	15 (71)	10 (48)	11 (52)	1.0	0.009	0.02
	Non-Gln ⁷⁰	47	6 (13)	41 (87)	30 (64)	17 (36)	37 (79)	10 (21)			
	Met ⁹¹	19	2 (11)	17 (89)	8 (42)	11 (58)	11 (58)	8 (42)	1.0	0.29	0.25
	Non-Met ⁹¹	49	6 (12)	43 (88)	28 (57)	21 (43)	36 (73)	13 (27)			

RVR = Rapid virological response; EVR = early virological response; ETR = end-of-treatment response; IRRDR = interferon/ribavirin resistance-determining region; Ala²³⁶⁰ = alanine at position 2360; ISDR = interferon sensitivity-determining region; Arg⁷⁰ = arginine at position 70; Leu⁹¹ = leucine at position 91;

Gln⁷⁰ = glutamine at position 70; Met⁹¹ = methionine at position 91.

^a Total number of isolates with a given factor. ^b Number of RVR, non-RVR, EVR, non-EVR, ETR or non-ETR cases with a given factor. ^c Values in parentheses are percentages.

Table 5. Correlation between NS5A and core protein polymorphisms

Factor	% (number of subjects/number of subtotal) ^a		p value
	IRRDR $\geq$ 6	IRRDR $\leq$ 5	
Ala ²³⁶⁰	50 (12/24)	14 (6/44)	0.003
Non-Ala ²³⁶⁰	50 (12/24)	86 (38/44)	
ISDR $\geq$ 2	42 (10/24)	18 (8/44)	0.047
ISDR $\leq$ 1	58 (14/24)	82 (36/44)	
Wild-core (Arg ⁷⁰ /Leu ⁹¹)	67 (16/24)	39 (17/44)	0.04
Non-wild-core	33 (8/24)	61 (27/44)	
Gln ⁷⁰	21 (5/24)	36 (16/44)	0.27
Non-Gln ⁷⁰	79 (19/24)	64 (28/44)	

IRRDR = Interferon/ribavirin resistance-determining region; Ala²³⁶⁰ = alanine at position 2360; ISDR = interferon sensitivity-determining region; Arg⁷⁰ = arginine at position 70; Leu⁹¹ = leucine at position 91; Gln⁷⁰ = glutamine at position 70.

^a Number of isolates with a certain factor/total number of HCV isolates with IRRDR $\geq$ 6 or IRRDR $\leq$ 5.

Correlation between NS5A and Core Polymorphisms

We then examined the possible correlation among the polymorphic factors in NS5A and core proteins. A significant correlation was observed between IRRDR $\leq$ 5 and non-Ala²³⁶⁰ as the majority (86%) of HCV isolates with IRRDR $\leq$ 5 had non-Ala²³⁶⁰ ($p = 0.003$) (table 5). Also, a significant correlation was obtained between IRRDR $\leq$ 5 and ISDR $\leq$ 1 since 82% of IRRDR $\leq$ 5 were ISDR $\leq$ 1 ($p = 0.047$). When IRRDR and core polymorphisms were compared, IRRDR $\geq$ 6 was significantly correlated with wild-core (Arg⁷⁰/Leu⁹¹) ($p = 0.04$). On the other hand, there was no significant correlation between IRRDR $\geq$ 6 and non-Gln⁷⁰, or IRRDR $\leq$ 5 and Gln⁷⁰, although the majority (79%) of IRRDR $\geq$ 6 were non-Gln⁷⁰.

Influence of NS5A and Core Polymorphisms on HCV Clearance Kinetics during PEG-IFN/RBV Combination Therapy

To investigate the influence of NS5A and core polymorphisms on HCV-RNA kinetics during the entire course of PEG-IFN/RBV combination therapy, Kaplan-Meier HCV survival curve analysis was carried out based on HCV-RNA positivity according to NS5A and core

Fig. 2. Kaplan-Meier HCV survival curve analysis based on HCV-RNA positivity during the whole treatment course according to NS5A (**a**, **b**) and the core protein (**c**, **d**) polymorphisms. The difference between the analyzed groups was measured by the log-rank test.

polymorphisms. The result showed that HCV isolates of IRRDR ≥ 6 were cleared from patients' sera more rapidly than those with IRRDR ≤ 5 (fig. 2a). On the other hand, HCV-RNA clearance kinetics did not differ significantly between HCV isolates of ISDR ≥ 2 and those of ISDR ≤ 1 (fig. 2b). As for the core polymorphism, HCV isolates of non-wild-core or Gln⁷⁰ persisted in patients' sera for longer periods of time than those of wild-core (Arg⁷⁰/Leu⁹¹) or non-Gln⁷⁰ (fig. 2c, d).

Next, HCV clearance kinetics during the very early stages of the treatment course, e.g., 24 h, 1, 2 and 4 weeks

after initiation of PEG-IFN/RBV therapy was examined. For this purpose, a possible correlation between the degree of IRRDR, ISDR and core polymorphisms and the proportion of patients who achieved significant reduction (1 log after 24 h, 1 log after 1 week, 1.5 log after 2 weeks, and 2 log after 4 weeks) of core antigen titers was analyzed. Interestingly, IRRDR ≥ 6 was significantly associated with reduction and/or disappearance of serum HCV core antigen titers at 24 h, 1, 2 and 4 weeks after initiation of the treatment (table 6). Again, there was no significant correlation between ISDR sequence variation

Table 6. Correlation between the proportions of patients with rapid reduction of HCV core antigen titers and degree of NS5A and core protein polymorphisms

Protein	Criteria	Number of patients with significant reduction of HCV core antigen titers/number of total							
		24 h ^a (≥1 log) ^b	p value	1 week (≥1 log)	p value	2 weeks (≥1.5 log)	p value	4 weeks (≥2 log)	p value
NS5A	IRRDR≥6	20/23	0.0006	18/23	0.004	17/23	0.018	19/23	0.008
	IRRDR≤5	17/40		16/40		16/40		19/40	
	ISDR≥2	10/19	1.0	11/19	0.59	10/19	1.0	11/19	1.0
	ISDR≤1	24/44		21/44		22/44		27/44	
Core	Wild core (Arg ⁷⁰ /Leu ⁹¹)	23/31	0.01	22/31	0.02	20/31	0.13	24/31	0.005
	Non-wild-core	13/32		13/32		14/32		13/32	
	Gln ⁷⁰	6/19	0.03	5/19	0.01	6/19	0.06	6/19	0.004
	Non-Gln ⁷⁰	28/44		27/44		26/44		32/44	

Note: Patients Nos. 108, 111, 129, 135 and 152 were excluded from this analysis because their core antigen titers at certain time points were missing.

IRRDR = Interferon/ribavirin resistance-determining region; ISDR = interferon sensitivity-determining region; Arg⁷⁰ = argi-

nine at position 70; Leu⁹¹ = leucine at position 91; Gln⁷⁰ = glutamine at position 70.

^a Period after initiation of IFN/RBV therapy.

^b Criteria of significant reduction of HCV core antigen titers.

(ISDR≥2 and ISDR≤1) and reduction of HCV core antigen titers during the very early stages of PEG-IFN/RBV therapy. On the other hand, non-wild-core or Gln⁷⁰ were significantly associated with slow reduction and/or persistence of HCV core antigen in the patients' sera (table 6).

Identification of Independent Predictive Factors for SVR by Uni- and Multivariate Logistic Regression Analyses

Finally, in order to identify significant independent predictive factors of PEG-IFN/RBV treatment outcome, first, all available data of baseline patients' parameters, on-treatment responses and NS5A and core polymorphisms were entered in a univariate logistic analysis. This analysis yielded 11 factors that were correlated or nearly correlated with the treatment outcome; IRRDR mutations categorized as IRRDR≥6 and IRRDR≤5, Ala²³⁶⁰ and non-Ala²³⁶⁰, core protein polymorphism categorized as wild-core (Arg⁷⁰/Leu⁹¹) and non-wild-core, Gln⁷⁰ and non-Gln⁷⁰, RVR and non-RVR, EVR and non-EVR, ETR and non-ETR, HCV core antigen titers, age, platelets count and hemoglobin levels (table 7). Subsequently, these 11 factors were entered in multivariate logistic regression analysis. This analysis yielded IRRDR mutations ($p = 0.005$), EVR ($p = 0.0001$) and age ($p = 0.02$) as independent predictive factors of PEG-IFN/RBV treatment outcome (table 7).

Discussion

Both host and viral genetic polymorphisms influence the outcome of PEG-IFN/RBV therapy for HCV-infected patients [15]. It has recently been reported that host genetic polymorphisms near or within the IL28B gene on chromosome 19 show a significant impact on the treatment outcome for patients infected with HCV genotype 1 (HCV-1a and -1b) [16–18]. Also, HCV genetic polymorphisms have been known to contribute to differences in the treatment outcome, as demonstrated by the observations that SVR rates for patients infected with HCV genotypes 2 and 3 are higher than those for patients infected with HCV genotype 1 [15]. Moreover, viral genetic polymorphisms, especially in the NS5A (ISDR and IRRDR) and the core regions, among HCV isolates of a given genotype have been linked to the difference in SVR rates [6–9, 19, 20]. In the present study, we compared the impact of IRRDR, ISDR and core polymorphisms of HCV-1b isolates on the clinical outcome of PEG-IFN/RBV therapy. Our results suggest that the degree of IRRDR mutations is more dominant than that of ISDR mutations and core polymorphism for predicting the anti-HCV treatment outcome.

IRRDR corresponds to a region near the C-terminus of NS5A. The obtained result that the IRRDR polymorphism influences the clinical outcome of IFN-based anti-HCV therapy can be linked to a recent experimental observation by Tsai et al. [21]. They reported that an HCV

Table 7. Uni- and multivariate logistic regression analyses to identify independent predictive factors for success of PEG-IFN/RBV combination therapy

Univariate variable	p value	Multivariate	
		odds ratio (95% CI)	p value
IRRDR mutations (IRRDR $\geq$ 6 vs. IRRDR $\leq$ 5)	<0.0001	14.33 (2.24–91.65)	0.005
Ala ²³⁶⁰	0.01	1.75 (0.19–15.36)	0.62
Core polymorphism (wild-core vs. non-wild-core)	0.06	0.41 (0.05–3.28)	0.34
Gln ⁷⁰	0.04		
RVR	<0.0001		
EVR	<0.0001	41.83 (6.12–285.68)	0.0001
ETR	<0.0001		
HCV core antigen, fmol/l	0.05		
Age	0.01	0.91 (0.84–0.99)	0.02
Platelets, $\times 10^4/\text{mm}^3$	0.07		
Hemoglobin, g/dl	0.006		

IRRDR = Interferon/ribavirin resistance-determining region; Ala²³⁶⁰ = alanine at position 2360; Gln⁷⁰ = glutamine at position 70; RVR = rapid virological response; EVR = early virological response; ETR = end-of-treatment response.

subgenomic RNA replicon containing NS5A of HCV-1b exerted more profound inhibitory effects on IFN activity than the original HCV-2a replicon, and that domain swapping between NS5A sequences of HCV-1b and -2a in the V3 and/or a C-terminus region including IRRDR resulted in a transfer of their anti-IFN activity. Since the C-terminal region of NS5A is among the most variable sequences across the different genotypes and subtypes of HCV [22], the difference in IFN responsiveness among different strains of a given HCV subtype could also be attributable, at least partly, to the genetic polymorphism within this region. The molecular mechanism underlying the possible involvement of IRRDR in IFN responsiveness of the virus is still unknown. The significant difference in IRRDR sequence pattern may suggest genetic flexibility of this region and, indeed, the C-terminal portion of NS5A was shown to tolerate sequence insertions and deletions [23, 24]. This means that the C-terminal portion of NS5A is not essential for virus replication in cultured cells. It does not exclude the possibility, however, that the same region plays an important role in modulating the interaction with various host systems, including IFN responsiveness. It is also possible that the genetic flexibility of this region, especially IRRDR, is accompanied by compensatory changes elsewhere in the viral genome and that these compensatory changes affect overall viral fitness and responses to IFN therapy [25].

While we observed significant correlation between the overall number of mutations in IRRDR and PEG-IFN/RBV responsiveness, we also found a particular aa mutation, Ala²³⁶⁰, that was significantly associated with SVR (tables 3, 7; fig. 1). It is possible that Ala or Val at this position confers a certain advantage for interaction between NS5A and the other viral or host proteins, which might affect IFN-induced antiviral responses. This issue needs to be elucidated in further studies.

The ISDR polymorphism was the only virological factor examined that showed a significant correlation with RVR (table 4), with the result being consistent with a recent report by other investigators [26]. This significant correlation, however, disappeared as the treatment went on. In contrast, the IRRDR polymorphism did not correlate significantly with RVR, however, it was the dominant viral genetic factor that was correlated with SVR (tables 3, 7). Interestingly, the combination of IRRDR and ISDR polymorphisms (IRRDR $\geq$ 6 *plus* ISDR $\geq$ 2) was significantly correlated with RVR and SVR ($p = 0.0001$ and 0.01 , respectively; data not shown). This suggests a possible integrated influence of IRRDR and ISDR polymorphisms, or NS5A as a whole, on the treatment outcome. Further study is needed to clarify the issue.

The core protein polymorphisms (wild-core vs. non-wild-core, and Gln⁷⁰ and non-Gln⁷⁰) were significantly correlated with the on-treatment HCV clearance kinetics

(fig. 2c, d; tables 4, 6). However, this significant correlation became blurred thereafter and eventually no significant correlation was observed between wild-core (Arg⁷⁰/Leu⁹¹) and the final treatment outcomes (table 3). On the other hand, Gln⁷⁰ was significantly associated with null-response, and almost significantly with non-SVR. This result is consistent, at least partly, with previous reports, including a recent multicenter study in Japan, that identified Gln⁷⁰ as a predictive factor for poor responses to PEG-IFN/RBV treatment [8, 9, 14].

Recently, it was reported that the C-terminal region of NS5A plays a critical role in regulating the early phase of HCV particle formation [27, 28]. Moreover, sequence alteration within this region affected the degree of interaction between NS5A and core protein, which in turn affected the efficiency of progeny virus production [29]. In the present study, we observed a significant correlation between the degree of IRRDR mutations (IRRDR $\geq$ 6) and the core polymorphism (table 5). Therefore, it would be interesting to investigate the degree of interaction between NS5A with IRRDR of high or low degrees of sequence variation and the wild-type (Arg⁷⁰/Leu⁹¹) or non-wild-type of core protein, and also the impact of these interactions on progeny virus production and IFN sensitivity of the virus.

The present study identified the IRRDR polymorphism as the only viral genetic factor that independently

predicted PEG-IFN/RBV treatment outcome (table 7). On the other hand, HCV is likely to utilize an alternative mechanism(s) by which to escape IFN actions through its various structural and non-structural proteins [30]. Also, a different lineage(s) of HCV-1b strains that relies more on the alternative mechanism than on IRRDR may prevail in other regions of the world. It is possible, therefore, that the impact of the IRRDR polymorphism differs with different cohorts. Analysis in a large-scale multicenter study is needed to clarify this issue.

In conclusion, NS5A (IRRDR and ISDR) and core protein polymorphisms are useful viral markers for predicting the outcome of PEG-IFN/RBV therapy for chronic hepatitis C. In particular, IRRDR $\geq$ 6 is a useful marker for prediction of SVR.

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